

Remarks/Arguments

Claims 17-32 are pending. Claims 17, 25, and 29 are amended to specify that the targeting vector includes homology arms directing the targeting vector to a pre-selected chromosomal location. The phrase "pre-selected chromosomal location" is identical in scope with the phrase "specific chromosomal location." The amendment has been made not for reasons related to patentability, but because the term "pre-selected" underscores that the specific chromosomal location has been pre-selected. Support for the amendments to the claims can be found in the claims as filed. No new matter has been added.

Rejections under 35 USC §103(a)

Claims 17-32 were rejected as being unpatentable over Rohozinski *et al.* (Genesis, 2002, 32:1-7), in view of Tsirigotis *et al.* (BioTechniques, 2001, 31:120-130) and Ghazizadeh *et al.* (J. of Investigative Dermatology, 1998, 111:492-496). This rejection is respectfully traversed.

The invention as claimed

Claims 17-20 are drawn to an *in vitro* method of directing a targeting vector to a pre-selected chromosomal location within a genome of a mouse embryonic stem (ES) cell, comprising introducing into the cell a targeting vector, wherein the targeting vector comprises a drug resistance gene under control of a ubiquitin promoter and homology arms directing the targeting vector to a pre-selected chromosomal location.

Claims 21-24 are drawn to the targeting vector used in the method of the invention. Claims 25-28 are drawn to an *in vitro* method of increasing targeting frequency in mouse embryonic stem (ES) cells, comprising introducing into a mouse ES cell a targeting vector, wherein the targeting vector comprises a drug resistance gene under control of a ubiquitin promoter, and homology arms directing the targeting vector to a pre-selected chromosomal location.

Claims 29-32 are drawn to an *in vitro* method of increasing the number of mouse embryonic stem (ES) cells correctly targeted with a targeting vector, comprising introducing into a mouse ES cell a targeting vector, wherein the targeting vector comprises a drug resistance gene under control of a ubiquitin promoter, and homology arms directing the targeting vector to a pre-selected chromosomal location.

The Cited References

Rohozinski *et al.* ("Rohozinski") describe manipulating the mouse Y chromosome by

homologous recombination in ES cells. Two genes on the mouse Y chromosome are targeted via insertional homologous recombination, with a targeting construct based on the 5' *hprt* phage vector, which uses a PGK promoter to drive expression of the neomycin resistance gene (see, for example, Zheng *et al.* (1999) *Nucleic Acids Res.* 27/11:2354-2360 at page 2355, col. 1, cited in Rohozinski *et al.*, included in the enclosed IDS). Rohozinski teaches using the phage vector having the PGK promoter because "it consistently gave very high rates of homologous recombination when targeting autosomal genes (15%-70%)" (see Rohozinski at page 2, col. 2, first paragraph), where four other conventional constructs having several different promoters resulted in no detectable targeting (see Rohozinski at page 2, col. 1, first full paragraph). Thus, Rohozinski teaches that a phage insertional vector comprising the PGK promoter is the best choice for targeting the Y chromosome in ES cells, but does not discuss targeting efficiency or targeting mouse ES cells with a targeting vector comprising a ubiquitin promoter. Rohozinski's teachings discourage the use of conventional replacement targeting vectors, motivate making changes to the electroporation buffer to improve targeting efficiency, and do not instruct as to the best promoter to use for the expression of the neomycin resistance gene.

Tsirigotis *et al.* ("Tsirigotis") describe transgenic ubiquitin expression in mice, driven by a ubiquitin promoter (the UbC promoter). Transgenic mice are made by (a) injecting 6xHis-ubiquitin/GFP DNA, operably linked to the UbC promoter, into oocyte pronuclei; (b) culturing the oocytes; and (c) implanting the cultured oocytes into pseudopregnant mice. Selection is achieved by assaying progeny tissue samples for UbC promoter-driven expression of ubiquitin fusion protein products (e.g., GFP). The UbC promoter is used because it is expected to drive transcription in a wide variety of tissues. Thus, Tsirigotis discloses making a transgenic mouse by a random (non-targeted) integration method, where the mouse expresses ubiquitin (in a fusion protein) in various tissues under the control of its own promoter (*i.e.*, the ubiquitin promoter), but does not discuss targeting efficiency or any targeting vector for mouse ES cells and does not discuss any promoter's activity at any pre-selected loci. Tsirigotis does not discuss using the UbC promoter to drive expression of a selection cassette for the purpose of selecting drug-resistant ES cells.

Ghazizadeh *et al.* ("Ghazizadeh") describe non-targeted integration into a pig genome using retrovirus vectors (*i.e.*, transduction). Although transduction of porcine keratinocytes is low using conventional retroviral vectors, Ghazizadeh teaches that retroviruses pseudotyped¹ with the VSV-G envelope protein can better transduce porcine keratinocytes (see, e.g., Ghazizadeh

¹ Retroviral pseudotyping is the packaging of an RNA genome of one retrovirus in the viral envelope of a different retrovirus.

at page 493, col. 2, last full paragraph, to page 494, cols. 1-2; page 496, col. 1, last full paragraph). Thus, Ghazizadeh discloses an improved method for random (non-targeted) integration into porcine keratinocytes (a single tissue type) by changing the retroviral envelope, and describes using neomycin phosphotransferase as a selectable marker, but does not discuss any factors concerning promoter selection, and never mentions ubiquitin promoters. Tsirigotis does not discuss targeting to any pre-selected locus.

The analysis under §103(a)

A careful analysis of the cited references shows that none of them, in any combination, discloses or suggests methods for targeting genes to a pre-selected locus in a genome using a targeting vector having a ubiquitin promoter operably linked to a drug resistance gene. None of the references suggest or motivate selecting a ubiquitin promoter to drive expression of a drug resistance gene (or, indeed, any other selectable marker) in a targeting vector for homologous recombination to direct a transgene to a pre-selected locus. None of the references discuss improving targeting efficiency by selecting a suitable promoter that is not silenced or inactivated at any pre-selected locus.

The Examiner asserts that Rohozinski teaches gene targeting in mouse ES cells by homologous recombination, although the Examiner conceded that Rohozinski does not teach using a targeting vector comprising a ubiquitin promoter. This deficiency of Rohozinski is purportedly addressed by Tsirigotis, which teaches that the ubiquitin promoter is one of the "best" promoters to achieve high levels of transgene expression in a tissue-independent manner, although Tsirigotis does not discuss vectors or promoters for targeted transgene insertion and does not discuss whether the ubiquitin promoter would be any more or less active than any other promoter at any specific pre-selected locus. Ghazizadeh, which also discusses only random (non-targeted) transgene insertion, allegedly provides motivation to use a drug resistance gene driven by the ubiquitin promoter to screen for cells expressing the integrated transgene. This combination of references allegedly contains all the limitations of the claimed vectors and methods and renders them obvious.

Applicants disagree with the Examiner's assertions, arguments, and conclusions regarding obviousness.

First, the Examiner has provided no reasonable scientific rationale or motivation to combine the cited references in the manner proposed.

Second, a fair reading of the references reveals that they should not be combined in the manner proposed, and that a person of ordinary skill would not be motivated to combine the

references.

Third, even if the references are combined as proposed by the Examiner, they do not add up to the present invention.

Fourth, the Examiner is using impermissible hindsight analysis in conjunction with an impermissible “obvious to try” standard to rationalize a motivation in the prior art for using a ubiquitin promoter in a targeting vector.

Finally, regardless of what the cited references disclose or motivate, Applicants’ invention of using a ubiquitin promoter in a targeting vector yields results that are unexpected. Further, the results obtained using the ubiquitin promoter in a targeting vector as claimed are unrelated to the Examiner’s assertions that the ubiquitin promoter is one of the “best” promoters because it achieves high levels of expression in a tissue-independent manner. The unexpected results include that the ubiquitin promoter can drive transcription at loci where other commonly used promoters are inactive or silenced. These results have nothing to do with what tissue is employed, or whether a promoter is active in one tissue or many tissues, but instead concerns promoter ability to drive transcription at a pre-selected locus.

The Examiner has not provided any reasonable basis to combine Rohozinski (which discloses insertional targeting vectors for mouse ES cells) with either Tsirigotis or Ghazizadeh (which both address random, non-targeted insertion of transgenes) in order to arrive at the claimed targeting vector. Neither Tsirigotis nor Ghazizadeh are concerned with targeting a transgene to a pre-selected locus—both of these references rely on random insertion of transgenes, so the transgene can insert at *any* suitable locus.

Tsirigotis employs the ubiquitin promoter to ensure that high levels of expression are exhibited across tissue types. There is no locus selection problem to address in Tsirigotis’s approach, since random integration eradicates issues associated with expression at a pre-selected locus such as, for example, promoter inactivation or silencing at the pre-selected locus.

The Ghazizadeh approach suggests no solutions regarding locus targeting either, since Ghazizadeh is concerned with non-targeted integration using retroviral vectors. To the extent that Ghazizadeh addresses any efficiency of transgene expression, it is concerned with swapping retroviral envelopes to achieve non-targeted transgene insertion.

Neither the approach of Tsirigotis nor the approach of Ghazizadeh bears any rational connection to targeting transgenes to a pre-selected locus employing a targeting vector, in a mouse ES cell or in any other cell. The random insertion of transgenes approach in Tsirigotis and Ghazizadeh would ensure that neither of these authors would even observe the problem of promoter inactivation or silencing at a pre-selected locus, since using the random approach the

transgenes will only be detected at loci where promoters driving selection are active. Thus, a person of ordinary skill would have no motivation whatsoever to look to either Tsirigotis or Ghazizadeh to arrive at new or different targeting vectors for targeting a transgene to a pre-selected locus, since neither reference could possibly shed any light on promoter inactivation or silencing at a pre-selected locus.

A fair reading of the cited references reveals that there is no basis to combine them as proposed by the Examiner. A person of ordinary skill would have no basis to modify Rohozinski in the manner proposed by the Examiner. Rohozinski teaches that using a phage-based insertional vector in combination with using spermidine in electroporation buffer solved their problems with targeting a pre-selected locus on the Y chromosome. There is no motivation or suggestion in Rohozinski that using a promoter active in a wide variety of tissues would help target the Y chromosome, particularly where Rohozinski teaches that such problems are effectively solved by using an insertional vector and modifying electroporation conditions. Therefore there is no motivation to look to Tsirigotis.

Similarly, there is nothing in Ghazizadeh that would help solve any problems faced by Rohozinski, since the solutions offered by Rohozinski are impossible to employ in the retroviral pseudotyping method of Ghazizadeh. Further, the claimed invention concerns none of the solutions disclosed by any of the cited references. And there are no shortcomings in the solutions expounded by Rohozinski that are addressed by either Tsirigotis or Ghazizadeh.

Moreover, Rohozinski's teachings counsel against consulting a reference such as Tsirigotis in the manner propounded by the Examiner. Rohozinski teaches that a PGK promoter-based selection cassette driving neo^r expression was found to be the **best choice** to target a pre-selected locus on the mouse Y chromosome. The inventors of the pending claims directly contradict this teaching. The inventors teach how to make and use an improved targeting vector that can successfully select properly targeted cells at loci where the PGK promoter is not only **not** the best choice, but where using a PGK promoter-driven cassette is **contraindicated**. Thus, while Rohozinski teaches that a PGK-based selection cassette is the best out of at least four different promoters tested, the inventors teach a targeting vector that should be used *in place of* a PGK promoter-based selection cassette. Neither Tsirigotis nor Ghazizadeh contravene or otherwise address the teaching of Rohozinski that the mouse Y chromosome is most effectively targeted with an insertional targeting vector having a PGK promoter-driven selection cassette. Accordingly, there is no scientifically rational route to be taken from Rohozinski in combination with any of the cited references that would lead a person of ordinary skill to the pending claims. Rohozinski is simply the wrong starting point, regardless

of its combination with any other reference.

Ghazizadeh teaches “a porcine organ culture model and manipulations required for *in situ* transduction using retroviral vectors” (Ghazizadeh at page 492, col. 2, last sentence of first paragraph). Ghazizadeh discloses no problems associated with random insertion of a transgene that comprises a neomycin resistance gene (neo^r) under the control of an RSV promoter (see, e.g., Ghazizadeh at page 493, col. 1, third full paragraph) that are not remedied by retroviral pseudotyping (see, e.g., Ghazizadeh at page 493, col. 2, last paragraph; page 496, last paragraph). Thus, Ghazizadeh teaches solving problems with gene transfer by retroviral vector pseudotyping, not selecting a good promoter for an improved targeting vector. Ghazizadeh, which teaches retroviral pseudotyping to solve random integration issues, cannot reasonably be combined with Rohozinski, which teaches using PGK promoter-driven selection to target a pre-selected locus in a mouse Y chromosome. Ghazizadeh would not be consulted to supplement the teachings of Tsirigotis, because Ghazizadeh focuses on random integration into a single tissue type, whereas Tsirigotis seeks to randomly insert transgenes into a wide variety of tissue types.

No rational basis exists to combine Tsirigotis with either Rohozinski or Ghazizadeh, because on the one hand Rohozinski teaches that PGK promoter-driven selection is preferred for targeting a pre-selected locus on the Y chromosome, and on the other hand Ghazizadeh teaches that an RSV promoter-driven expression of the neo^r gene is suitable for randomly inserting transgenes into a specific tissue type (*i.e.*, pig skin). If Rohozinski is to be believed, the PGK promoter is best for driving expression of a selectable marker in a targeting vector, so there is no need to consult Tsirigotis or Ghazizadeh for a ubiquitin promoter, particularly where Rohozinski discloses that the PGK promoter was the best of five promoters tested and Ghazizadeh discloses that RSV promoter-driven expression works just fine.

Even if all the references are combined, they do not disclose, teach, or suggest a targeting vector comprising a drug resistance gene under control of a ubiquitin promoter and homology arms directing the targeting vector to a pre-selected chromosomal location, or methods of using such a vector. None of the references alone or in combination discloses or suggests that screening for transgenic cells having a transgene properly targeted to a pre-selected locus can be facilitated by placing a drug resistance gene under control of a ubiquitin promoter. None of the references alone or in combination articulate the importance of selecting a suitable promoter that is not inactivated or silenced when faced with inserting a transgene at a pre-selected locus.

Applicants submit that the Examiner’s assertions that the claimed vector is obvious relies

on an “obvious to try” argument. The Examiner’s argument amounts to the following: if difficulty is encountered in obtaining transgenic cells using a targeting vector that has a selectable marker driven by a PGK promoter according to Rohozinski, a person of ordinary skill would try another promoter. Tsirigotis discloses that the ubiquitin promoter exhibits high expression in a variety of tissues, so a person of ordinary skill should obviously try the ubiquitin promoter. Because the ubiquitin promoter is expressed in a wide variety of tissues, the argument continues, then there is a likelihood of success in using the ubiquitin promoter. But this reasoning contains serious flaws.

First, this reasoning urges a person of ordinary skill seeking to introduce a transgene at a pre-selected locus to try promoters, such as the ubiquitin promoter, that are constitutively expressed in a wide variety of tissues. There exists a very large plurality of such promoters. Genes that are constitutively expressed in a wide variety of tissues are commonly called “housekeeping genes.” As reflected in Zhang *et al.* (2004) *Mol. Biol. Evol.* 21(2):236-239 (attached hereto in an information disclosure statement), there are at least about 350 to well over 500 such housekeeping genes in a mammalian genome, and thus at least 350 to well over 500 promoters to “try.” With such an expansive selection, the cited references offer no guidance as to which constitutively active promoter to select. Nothing in the cited references suggests that the ubiquitin promoter—just one of the about 350 to well over 500 constitutively active promoters in a wide variety of tissues—is any better than a promoter for any other housekeeping gene. Indeed, without the benefit of hindsight and Applicants’ disclosure in hand, there is no guidance in the art to select the ubiquitin promoter to drive expression at a pre-selected locus where another promoter (e.g., the PGK promoter) is observed to be inactive. In contrast, the cited references teach that transgene insertion problems are solved by manipulations like changing components of electroporation buffer, use a pseudotyped retrovirus, etc. None of the references even hint that targeting problems might be a reflection of promoter silencing at a pre-selected locus, such that even if a transgene is properly inserted at the pre-selected locus, it is not observed using promoter-driven selection methods because the promoter is silenced at that locus.

Second, this reasoning is based on a flawed syllogism. The flawed syllogism is this: if I do not obtain drug resistant clones when I am trying to insert a transgene with a drug selection cassette driven by promoter 1 at a pre-selected locus, I need to try another promoter that exhibits a high level of expression in a wide variety of tissues; ubiquitin is a promoter that exhibits a high level of expression in a wide variety of tissues; therefore, if I replace promoter 1 with the ubiquitin promoter or another promoter (promoter 2) that exhibits a high level of

expression in a wide variety of tissues, then the drug selection marker will express at the pre-selected locus. The syllogism is flawed because the ability of a promoter to be active in a wide variety of tissues has no bearing on whether that promoter is active or silenced at a specific, pre-selected locus. Thus, any indication in the art that the ubiquitin promoter is active in a wide variety of tissues does not motivate its use in a targeting vector. There is nothing in the cited references that support the proposition that a promoter active in a wide variety of tissues would also be active at any given pre-selected locus, and the Examiner has not articulated that such a proposition exists in the art. And the Examiner never explained why there would be any expectation of success whatsoever in using a ubiquitin promoter to drive expression at a locus that silences another commonly used promoter (e.g., the PGK promoter). Thus, the art provides no suggestion for using a ubiquitin promoter in a targeting vector to insert a transgene at a pre-selected locus.

Finally, using a ubiquitin promoter in a targeting vector yields results unexpected and unrelated to the assertion that the ubiquitin promoter is one of the “best” promoters to achieve high levels of transgene expression in a tissue-independent manner. According to the Examiner’s argument, the “best” promoter to use to drive expression of a selectable marker in a targeting vector to target a transgene to a pre-selected locus is a promoter that is active in a wide variety of tissues (such as the ubiquitin promoter). It is known in the art that the PGK promoter, like the ubiquitin promoter, is active in a wide variety of tissues. But the inventors have shown that a promoter that should be a “best” promoter (i.e., the PGK promoter, which, like ubiquitin, is active in a wide variety of tissues) is inactive or silenced at certain pre-selected loci. The inventors have shown that the PGK promoter, does *not* promote sufficient expression of the neo^r gene at the pre-selected loci to permit isolation of clones in which the targeting vector has inserted at the pre-selected locus. Logically, other promoters that are like the PGK promoter, that is, other promoters that are active in a wide variety of tissues, should also be inactive or silenced at the pre-selected loci. Applicants have shown that this is *not* the case. Applicants have shown that promoters which are active in a wide variety of tissues do not share the ability to drive expression at certain pre-selected loci (see, e.g., the specification at Table 2; see also the Frendewey declaration at Table A). It is a surprising and unexpected result that the ubiquitin promoter would drive expression at one or more loci where the PGK promoter is silenced. There is no teaching or suggestion in the art that there are loci where the PGK promoter is silenced but the ubiquitin promoter is *not* silenced.

This can be more readily understood following a review of how selectable markers function when used in targeting constructs.

One of the most widely used methods for screening cells that have taken up a transgene is to introduce the transgene with a selection cassette that includes a neomycin resistance gene (neo^r) driven by the PGK promoter. PGK-driven neomycin resistance facilitates identification of cells having the transgene because there is no need to directly inspect the genome for integration—if the cells contain the transgene, they contain the PGK-driven neomycin resistance gene, and so cells containing the transgene can be selected on the basis of acquired neomycin resistance. Since transgenic cells can be identified without resort to genotyping the locus (e.g., by sequencing, PCR, or hybridization assays), large numbers of cells can be screened efficiently and at relatively low cost based solely on expression of the neo^r gene product. But the PGK promoter-driven neomycin resistance approach depends on the ability of the PGK promoter to drive transcription of the neo^r gene inserted into at least one locus in the genome.

Where transgene integration is *random*, or non-targeted, the PGK promoter-driven neomycin resistance approach almost always works well because there is generally at least one locus in a genome where the PGK promoter is transcriptionally active and thus drives neo^r gene expression. But where transgene integration is targeted to a specific pre-selected locus (as in a targeting vector for homologous recombination), there is no guarantee that the PGK promoter will be transcriptionally active at the specific pre-selected locus. At a pre-selected locus where the PGK promoter is silenced, selection based on neo^r expression cannot be made because there is no neomycin resistant phenotype as the result of promoter silencing. At such a locus, large scale screening for targeted transgene insertion is not feasible, because it cannot be achieved based on a drug resistance phenotype. In such instances, genotyping the targeted locus by, for example, sequencing, PCR, or hybridization assays would be necessary to determine if the transgene (and the silenced PGK promoter) is present at the targeted locus. Genotyping methods are relatively time-consuming, expensive and cumbersome and, in most instances, render screening large numbers of cells for transgene integration unfeasible. The Standard procedure to screen for targeted clones is first to select for drug-resistant clones and then to use molecular assays, e.g., PCR, hybridization, to identify the correctly targeted clones. If at the pre-selected locus expression of neo^r from the PGK promoter is insufficient to permit drug-resistant growth, then none of the correctly targeted clones would survive and none would be detected by the molecular assays. Applicants' invention addresses this problem by providing a targeting vector for homologous recombination that can exhibit a drug resistance phenotype at a pre-selected locus that silences another promoter, e.g., a PGK promoter.

Without wishing to be bound by any particular theory, Applicants submit that the increase in targeting efficiency observed using the claimed targeting vector may at least in part

be due to the fact that the ubiquitin promoter is not inactivated or silenced at pre-selected loci where other promoters, e.g., the PGK promoter or the SV40 promoter, are silenced. This results in better targeting efficiency not because the ubiquitin promoter exhibits a higher level of activity at any given locus than any other promoter, but because it exhibits activity at loci where other promoters exhibit no activity or poor activity. The better targeting efficiency is reflected in the fact that out of the number of positive clones (*i.e.*, cells that exhibit drug resistance conferred by the selectable marker), the number of properly targeted transgenes is higher for the ubiquitin promoter than for the PGK promoter (see Table A, cols. 5 and 6 of the Frendewey declaration). Applicants submit that this phenomenon may reflect the fact that drug resistance can be conferred where multiple copies of a transgene's selectable marker are inserted, sometimes in improper orientation, thereby resulting in clones that overcome a locus's silencing or inactivation effect. But many of these multi-copy insertions do not contain the transgene in the desired orientation at the pre-selected locus. Indeed, Applicants have found that at loci where the targeting efficiency of vectors employing a PGK promoter is low in comparison to using a ubiquitin promoter, a majority—if not all—of the transgenes introduced with the vector having the PGK promoter are present as multi-copy inserts, whereas in such situations the median copy number at the same locus using a targeting vector having the ubiquitin promoter is about one. Thus, many more clones that survive drug selection must be screened if the PGK promoter has been used in the targeting vector, in comparison to fewer clones to screen if the ubiquitin promoter has been used in the targeting vector. This results in decreased targeting efficiency (as reflected by the number of clones bearing the transgene at a desired orientation and copy number compared to the total number of drug resistant clones) when using the PGK promoter at certain loci. Accordingly, employing the ubiquitin promoter in a targeting construct increases targeting efficiency at certain pre-selected loci.

Applicants provide a novel solution to targeting transgenes to pre-selected loci, including solutions for targeting transgenes to loci where other promoters are not transcriptionally active. Applicants' invention includes a targeting vector for homologous recombination in mouse ES cells that allows transgenes to be screened by drug resistance phenotype where the targeting is to pre-selected loci where at least one commonly employed vector in the art is unable to drive sufficient expression of a drug resistance gene.

For the reasons stated above, Applicants submit that the claims are not obvious in light of the cited references, and request reconsideration and withdrawal of the obviousness rejections.

Conclusion

It is believed that this document is fully responsive to the Office action dated 28 August 2006. It is believed that the claims are now in condition for allowance, and such action is respectfully urged.

Fees

Applicants submit that no fee other than the fee for a three-month extension of time and the IDS fee are due. If any further fees are due, or overpayment has been made, please charge, or credit, Deposit Account No. 18-0650 in the amount of the overpayment or fee deficiency.

Respectfully submitted,


Valeta Gregg, Reg. No. 35,127
Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591
Direct Tel.: (914) 593-1077